Chapter 2
Equipment

Sample Processing Apparatus

- Centrifuge, with swinging bucket rotors having a capacity of 15 to 250 mL or larger per conical tube or bottle.
- Mixer, vortexer.
- Vacuum source.
- Membrane filter holder, Hoefer manifold, model FH 225V[1], 10 place holder for 25 mm diameter filters.
- Slide warming tray, or incubator, 37°C.
- pH meter.
- Rubber policeman.
- Stomacher Lab Blender, model 3500 (BA 7022)[2] (optional).

Sample Examination Apparatus

- Microscope, capable of epifluorescence and differential interference contrast (D.I.C.) or Hoffman modulation® optics, with stage and ocular micrometers and 20X (N.A. = 0.6) to 100X (N.A. = 1.3) objectives. Equip the microscope with appropriate excitation and band pass filters for examining fluorescein isothiocyanate-labeled specimens (exciter filter: 450-490 nm; dichroic beam-splitting mirror: 510 nm; barrier or suppression filter: 515-520 nm).

Reagents and Materials

Purity of Reagents

Reagent grade chemicals shall be used in all tests. Unless otherwise indicated, it is intended that all reagents shall conform to the specifications of the committee on Analytical Reagents of the American Chemical Society where such specifications are available[3].

Preparation of Reagents

Prepare reagents as specified by the formulations.

Purity of Water

Use distilled deionized, double distilled, or reagent grade water.

Sample Collection Reagents

- Sodium Thiosulfate Solution (2.0%) - Dissolve 2.0 g of sodium thiosulfate ($Na_2S_2O_3 \cdot 5H_2O$) in 50 mL water and then adjust to a final volume of 100 mL.

Sample Processing Reagents

- Neutral Buffered Formalin Solution (10%) - Dissolve 0.762 g disodium hydrogen phosphate (Na_2HPO_4), 0.019 g sodium dihydrogen phosphate (NaH_2PO_4), and 100 mL formalin in water to a final volume of 1 L.

- Phosphate Buffered Saline (PBS) - Prepare a 10X stock solution by dissolving 80 g sodium chloride (NaCl), 2 g potassium dihydrogen phosphate (KH_2PO_4), 29 g hydrated disodium hydrogen phosphate ($Na_2HPO_4 \cdot 12\ H_2O$) and 2 g potassium chloride (KCl) in water to a final volume of 1 L. The 10X solution is used to prepare 1X PBS by diluting one volume of the 10X solution with 9 volumes of water and adjust the pH with a pH meter to 7.4 with 0.1 N HCl or 0.1 N NaOH before use.

- Sodium Dodecyl Sulfate Stock Solution (1%) - Prepare solution by dissolving 1.0 g of sodium dodecyl sulfate (SDS) in water to a final volume of 100 mL.

- Tween 80 Stock Solution (1%) - Mix 1.0 mL of polyoxyethylenesorbitan monooleate 80 (Tween 80) stock solution with 99 mL of water.

- Eluting Solution (Buffered Detergent Solution) - Prepare solution by mixing 100 mL 1% SDS, 100 mL 1% Tween 80, 100 mL 10X PBS, and 0.1 mL Sigma Antifoam A (Cat. # A 5758) with 500 mL water. Adjust the pH to 7.4 using a pH meter. Adjust the final volume to 1 L with additional water. Use within one week of preparation.

- Sucrose Solution (2.5 M) - Dissolve 85.58 g of sucrose in 40 mL prewarmed water then adjust the final volume to 100 mL with water.

- Percoll-Sucrose Flotation Solution, Sp. Gr. 1.10 - Mix 45 mL Percoll (sp. gr. 1.13; Sigma Cat. # P 1644), 45 mL water and 10 mL 2.5 M sucrose solution. Check the specific gravity with a hydrometer. The specific gravity should be between 1.09 and 1.10 (do not use if less than 1.09). Store at 4°C and use within a week. Allow to reach room temperature before use.

Sample Examination Reagents

- Ensys Hydrofluor-Combo kit[4] for detecting *Giardia* cysts and *Cryptosporidium* oocysts in water samples. The expiration date for the reagents is printed on the Hydroflour-Combo kit label. Discard the kit once the expiration date is reached. Store the kit at 2-8°C and return it promptly to this temperature range after each use. The labeling reagent should be protected from exposure to light. Do not freeze any of the reagents in this kit. Diluted, unused working reagents should be discarded after 48 hours.

- Ethanol, (95%).

- Glycerol.

- Ethanol/Glycerol Series - Prepare a series of solutions according to the following table:

95% Ethanol	Glycerol	Reagent Water	Final Volume	Final % Ethanol
10mL	5 mL	80 mL	95 mL	10
20 mL	5 mL	70 mL	95 mL	20
40 mL	5 mL	50 mL	95 mL	40
80 mL	5 mL	10 mL	95 mL	80
95 mL	5 mL	0 mL	100 mL	90.2

- DABCO-Glycerol Mounting Medium (2%) - Prewarm 95 mL glycerol using a magnetic stir bar on a heating stir plate. Add 2 g 1,4 diazabicyclo [2.2.2] octane (DABCO, Sigma #D-2522) to the warm glycerol with continuous stirring until it dissolves. (**CAUTION: hygroscopic; causes burns; avoid inhalation, as well as skin and eye contact.**) Adjust the final volume to 100 mL with additional glycerol. Store at room temperature and discard after 6 months.

- Bovine Serum Albumin (1%) - Sprinkle 1.0 g bovine serum albumin (BSA) crystals over 85

mL 1X PBS, pH 7.4. Allow crystals to fall before stirring into solution with a magnetic stir bar. After the BSA is dissolved, adjust the volume to 100 mL with PBS. For prolonged storage, sterilize by filtering through a 0.22 µm membrane filter into a sterile tube or bottle. Store at 4°C and discard after 6 months.

Sample Collection Materials

- Filters and filter holder, either a 25.4 cm (10 in.) long 1 µm nominal porosity, yarn-wound polypropylene cartridge Commercial honeycomb filter tube (M39R10A; Commercial Filters Parker Hannifin Corp., P.O. Box 1300, Lebanon, IN) with a Commercial LT-10 filter holder or a 25.4 cm (10 in.) long 1 µm nominal porosity Filterite polypropylene cartridge (U1A10U; Filterite Corporation, Timmonium, MD), with a Filterite LMO10U-3/4 filter holder must be used.

- Garden hose or PVC tubing and connectors.
- Pressure regulator.
- Pressure gauge(s).
- Proportioner.
- Plastic sample bags, double-track, zipper-lock or equivalent, approximately 15 in. (38 cm) x 15 in (38 cm).
- Cold packs or wet ice.

Sample Processing Materials

- Pans or trays, stainless steel or glass trays, approx. 16.5 in. (41.91 cm) x 10 in. (25.4 cm) x 2 in. (5.08 cm) deep.

- Disposable knife/cutting tool, for cutting the polypropylene filter fibers off filter core.

- Hydrometer, for liquids heavier than water (range: 1.000-1.225), for adjusting specific gravity of flotation solutions.

Sample Examination Materials

- Slides, glass microscope, 1 in. (2.54 cm.) x 3 in. (7.62 cm) or 2 in. (5.08 cm.) x 3 in. (7.62 cm.).

- Cover slips, 25 mm^2, No. 1 1/2.

- Filters, Sartorius brand[5] cellulose acetate, 0.22 µm pore size, 25 mm diameter.

- Support Filters, ethanol-compatible membrane, any pore size, 25 mm.

- Fingernail polish, clear, or clear fixative (Cat. # 60-4890; PGC Scientifics[6]).

- Splinter forceps, fine tip.

- Blunt-end filter forceps.

End Notes

[1] Hoefer Scientific Instruments, 654 Minnesota Street, Box 77387, San Francisco, CA 94107

[2] Tekmar Company, P.O. Box 371856, Cincinnati, OH 45222-1856

[3] "Reagent Chemicals, American Chemical Society Specifications", American Chemical Society, Washington, DC. For suggestions on the testing of reagents not listed by the American Chemical Society, see "Annular Standards for Laboratory Chemicals," BDH, Poole, Dorset, U.K. and the "United States Pharmacopeia."

[4] Ensys Environmental Products, Inc., P.O. Box 14063, Research Triangle Park, NC 27709

[5] Sartorius Corp., Filter Div., 30940 San Clemente, Hayward, CA 94544

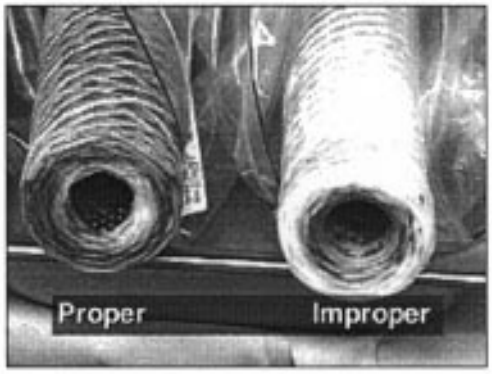
[6] PGC Scientifics, P.O. Box 7277, Gaithersburg, MD 20898-7277

Chapter 3
Filter Elution

Before beginning the assay procedure, check the validity and completeness of the data provided with the sample. Essential information includes the:

- Name of the source water sampled.
- Number of gallons filtered.
- Turbidity of the sampled source water.
- Time and date when the sample collection began.

This information is required to properly report the results of the analysis, and to ensure that the filter is eluted within 96 hours of the start of sampling.



Check the filter to ensure that the sample was collected properly. If the fibers closest to the filter core appear to be the dirtiest, the sampled water may have flowed through the filter in the wrong direction. If improper sampling is suspected, investigate the sampling procedures. An improperly collected sample must not be analyzed.

The initiation of sample collection and elution from the collection filter must be performed within 96 hours. Two approaches to eluting the particulates from the filter may be used: either washing by hand or using a stomacher.

The materials used from this point on are potentially of a biohazardous nature, and must be treated and discarded appropriately. Before starting the analysis, make sure you are wearing safety glasses and latex gloves.

Filter Washing

Step 1 Pour the residual solution in the bag into a beaker, rinse the bag with eluting solution, add the rinse solution to the beaker and discard the bag.

Handwashing

Step 1 Using a razor knife or other appropriate disposable cutting instrument, cut the filter fibers lengthwise down to the core. Discard the blade after the fibers have been cut.

Rinse the filter core with eluating solution into the beaker containing residual solution from the filter bag.

Divide the filter fibers into a minimum of six equal portions with one-sixth consisting of those cleanest fibers nearest the core; the second one-sixth being the second, slightly dirtier, layer of fibers, and so on to the final one-sixth consisting of the outer-most filter fibers (the dirtiest fibers).

Step 2 Beginning with the cleanest fibers (the one-sixth nearest the core), hand wash the fibers in three consecutive 1.0 L volumes of eluting solution.

Wash the fibers by kneading them in the eluting solution contained either in a beaker or a plastic bag.

Wring the fibers to express as much of the liquid as possible before discarding.

Maintain the three 1.0 L volumes of eluate separate throughout the washing procedure.

Step 3 Using the same three 1.0 L volumes of eluate used in Step 2, repeat the washing procedure on the second one-sixth layer of fibers and then on sequentially to the final outer one-sixth layer of fibers. The minimum total wash time of fibers should be 30 minutes.

! For extremely dirty filters, additional beakers of eluting solution may be required.

Step 4 After all the fibers have been washed, combine the three 1.0 L volumes of eluate with the residual filter water collected when the filter was unwrapped. Discard the filter fibers before preparing the eluate and residual water for concentration in a centrifuge.

Stomacher Washing

Step 1 Using a razor knife or other appropriate disposable cutting instrument, cut the filter fibers lengthwise down to the core. Discard the blade after cutting the fibers.

Rinse the filter core with eluating solution into the beaker containing residual solution from the filter bag.

Step 2 After loosening the fibers, place all the filter fibers in a stomacher bag that has a 3,500 mL capacity. To ensure against bag breakage and sample loss, place the stomacher bag that contains the filter fibers into a second stomacher bag.

Step 3 Add 1.75 L of eluting solution to the fibers, and homogenize for two 5-minute intervals. Between each homogenization period, knead the filter material by hand to redistribute the fibers in the bag.

Step 4 At the end of the second homogenization period, pour the eluted particulate suspension into a 4-liter pooling beaker. Wring the fibers out to express as much of the liquid as possible before.

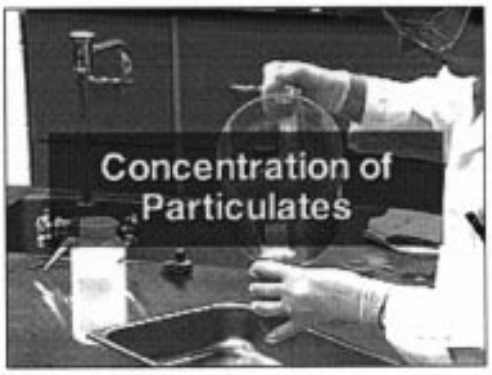
Step 5 Return the fibers to the stomacher bag and add 1.0 L of eluting solution. Homogenize, as in Step 3, for two 5-minute periods. Between each homogenization period, knead the fiber materials by hand to redistribute them in the bag.

Step 6 At the end of the second homogenization period, add the eluted particulate suspension to the 4-liter pooling beaker. Wring the fibers out to express as much of the liquid as possible into the beaker. Discard the fibers, and rinse the stomacher bag with eluting solution into the pooling beaker.

Concentration of Particulates

After the filter fibers have been washed, and the eluate and residual water has been pooled, the particulates in suspension must be recovered using a centrifuge.

Step 1 Pour the liquid into conical centrifuge bottles, then balance the bottles in their shields.



! Make sure to use the appropriate cushion for each bottle in the shield. Otherwise, the conical bottle will collapse during centrifugation.

Step 2 Concentrate the combined eluate and residual water into a single pellet by centrifugation at 1,050 x g for 10 minutes using a swinging bucket rotor and plastic conical centrifuge bottles.

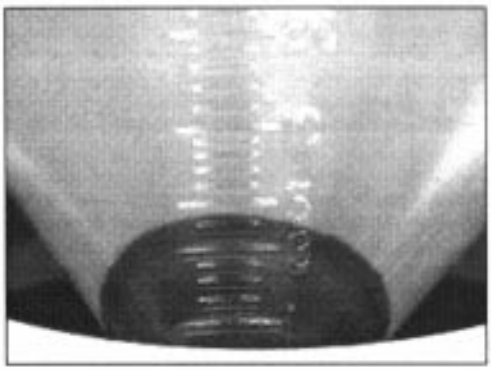
! You must use a swinging bucket rotor for this operation.

Step 3 At the end of 10 minutes, carefully aspirate and discard the supernatant fluid. Resuspend the pellet in sufficient elution solution by vortexing.

! Vortexing is done between centrifugations to prevent excessive packing of the particulates that form the pellet.

Step 4 Continue centrifugations of the pooled eluate and residual water at 1,050 x g for 10 minutes until all the particulates are concentrated in one conical bottle.

Step 5 At the end of this period, remove the bottle from the centrifuge and record the packed pellet volume.



Step 6 Carefully aspirate and discard the supernatant fluid. Resuspend the pellet by vortexing in an equal volume of 10% neutral buffered formalin solution. If the packed pellet volume is less than 0.5 mL, bring the pellet and solution volume to 0.5 mL with eluting solution before adding enough 10% neutral buffered formalin solution to bring the resuspended pellet volume to 1.0 mL.

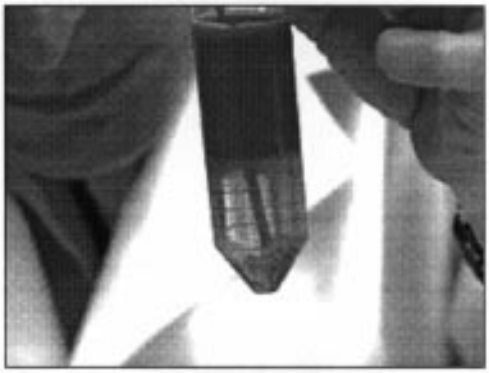
Step 7 At this point, a break of up to 72 hours may be inserted if the procedure is not going to progress immediately to the Flotation Purification procedure described in chapter 4. If a break is inserted at this point, be sure to store the formalin-treated sample at 4°C for not more than 72 hours.

Chapter 4
Flotation Purification

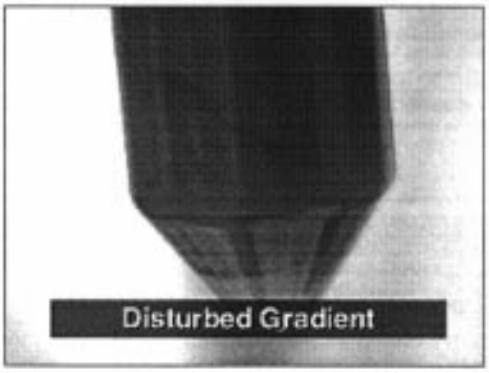
Flotation Purification is used to separate any *Giardia* cysts and *Cryptosporidium* oocysts present in the sample from certain other particulates and other debris which have a greater specific gravity.

Step 1 In one or more clear plastic 50 mL conical centrifuge tubes, as necessary, vortex a volume of resuspended pellet equivalent to not more than 0.5 mL of packed pellet volume with enough eluting solution to make a final volume of 20 mL.

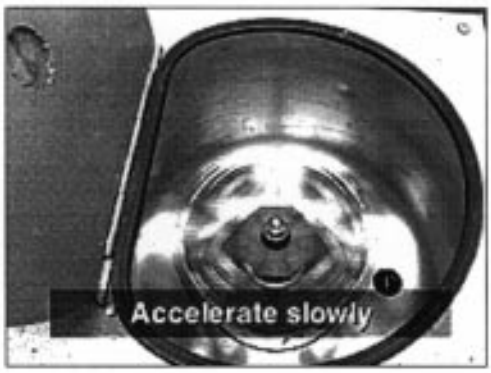
Step 2 Using a 50 mL syringe and 14 gauge cannula, underlay the 20 mL vortexed suspension of particulates with 30 mL Percoll-sucrose flotation solution (sp. gr. 1.1).



! Some water samples will have heavier particulates that penetrate the gradient before centrifugation begins in Step 3. This is normal. However, if for any reason the interface is disturbed and mixed, then another sample aliquot must be used.

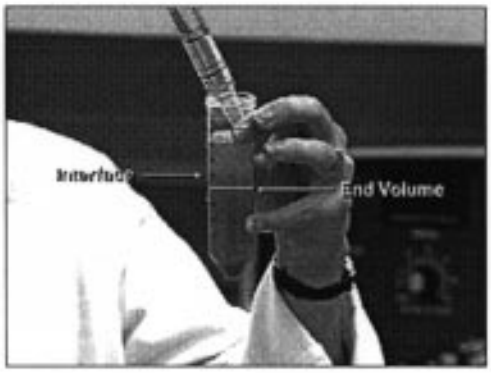


Step 3 Without disturbing the pellet suspension/Percoll-sucrose interface, centrifuge the preparation at 1,050 x g for 10 minutes using a swinging bucket rotor.



! To avoid disrupting the interface, slowly accelerate the centrifuge over a 30-second interval up to the speed where the tubes are horizontal. Similarly, at the end of centrifugation, decelerate slowly. **Do not use the brake!**

Step 4 Using a polystyrene 25 mL pipet rinsed with eluting solution, draw off the top 20 mL particulate suspension layer, the interface, and 5 mL of the Percoll-sucrose below the interface.



Place all these volumes in a plastic 50 mL conical centrifuge tube.

Step 5 Add additional eluting solution to the plastic conical centrifuge tube (Step 4) to bring the final volume to 50 mL. Centrifuge at 1,050 x g for 10 minutes.

Step 6 Aspirate and discard the supernatant fluid down to 5 mL (plus pellet).

Resuspend the pellet by vortexing, and save this suspension for further processing with fluorescent antibody reagents.